J. Clin. Chem. Clin. Biochem.
Vol. 28, 1990, pp. 217–219

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Berlin · New York

Direct Determination of Chloramphenicol Acetyltransferase (CAT) Activity in Homogenates of Transfected Mammalian Cells by High Performance Liquid Chromatography[1])

By *H. Siegert*[1], *B. Wittig*[2] and *S. Wölfl*[2]

[1] *Institut für organische Chemie, Sonderforschungsbereich 312, Freie Universität Berlin*
[2] *Institut für Molekularbiologie und Biochemie, Freie Universität Berlin*

(Received December 1, 1989)

Summary: The conversion of chloramphenicol to its monoacetylated form by homogenates of pRSV CAT-transfected mammalian cells was assayed by HPLC. The method differed from conventional procedures, in that the extraction of chloramphenicol and its acetylated forms into an organic solvent was replaced by treatment of the cell homogenates with acetonitrile. This allows a rapid, direct analysis by HPLC with few experimental steps.

Introduction

Since chloramphenicol acetyltransferase (EC 2.3.1.28) is not found in mammalian cells assay of its activity is used widely to monitor the transfection of mammalian cells by foreign DNA linked with the chloramphenicol acetyltransferase gene. Incubation of homogenates of such transfected cells in the presence of ^{14}C-labelled chloramphenicol and acetyl CoA leads to the formation of ^{14}C-labelled acetylchloramphenicol if chloramphenicol acetyltransferase is expressed. For the analysis of the reaction products, extraction with ethylacetate and subsequent thin layer chromatography were employed in the original published procedure (1). Alternatively, the extracted reaction products may be analysed by HPLC (2, 3), which makes it possible to work with unlabelled chloramphenicol. However, extraction of cell homogenates with ethylacetate often leads to variable recoveries of reaction products. This is probably due to the small volumes involved as well as to cell type specific differences in protein/lipid concentrations.

Therefore, the recently published method of deproteinizing the suspension with acetonitrile and subsequent direct analysis of the aqueous supernatant by HPLC offers a true advantage over the established procedure.

So far the 'acetonitrile procedure' has only been used to measure chloramphenicol acetyltransferase activity in chloramphenicol-resistent bacterial strains. In adapting the procedure for the direct analysis of chloramphenicol acetyltransferase in transfected mammalian cells, one well known difference had to be taken into account. Chloramphenicol-resistent bacteria produce enough chloramphenicol acetyltransferase enzyme to convert 30 mmol/min of chloramphenicol (4). In contrast, chloramphenicol acetyltransferase expression in mammalian cells is much less effective. Under the conditions we used, only 16 µmol/min of chloramphenicol became acetylated. We demonstrate in this paper that nevertheless such poor chloramphenicol acetyltransferase activities can be analysed by direct loading of the HPLC with acetonitrile-treated homogenates.

[1]) This paper contains in part data from the doctoral thesis of *H. Siegert*.

Materials and Methods

Mouse L-cells were grown non-confluently on 10 cm dishes in *Dulbecco*'s modified *Eagle* medium containing new-born calf serum, volume fraction 0.1, 37 °C, 7% CO_2 incubator.

Transfection was performed by calcium phosphate precipitation and glycerol shock treatment. 500 µl of 250 mmol/l $CaCl_2$ were added cautiously to 5 µg pRSV CAT DNA [1 g/l] (no mixing), underlayed with 500 µg 2 X Hepes buffered saline (280 mmol/l NaCl, 50 mmol/l Hepes, 1.5 mmol/l Na_2HPO_4), and gently mixed by blowing in a few bubbles with the pipettor. The suspension was stored for 30 min at room temperature and the mixture added to the cell medium, followed by incubation for 4 h. For glycerol-shock treatment the medium was replaced by 5 ml serum-free medium containing a volume fraction 0.15 of glycerol, incubated for 2 min, the 'glycerol medium' removed and the cells incubated for 48 h with normal medium.

Following incubation, the cells were washed three times with phosphate-buffered saline, harvested by scraping and resuspended in a mictrotube with 100 µl of 200 mmol/l Tris HCl (pH 7.2). L-cells were disrupted by 4 cycles of sonication (10 seconds each, with intervals of 30 seconds (Sonicator Heat Systems-Ultrasonic Inc. Model W-220F, microtip) and the debris pelleted. Simulated transfection experiments were carried out in the absence of DNA and defined amounts of purchased chloramphenicol acetyltransferase (Boehringer Mannheim GmbH) were added to the disrupted cells.

Chloramphenicol acetyltransferase activity was assayed under the following conditions. The test contained 20 µl of the cell supernatant (45 µl for simulated transfection experiments) in a volume of 145 µl (200 mmol/l Tris-HCl, pH 7.2), 20 µl acetylCoA (4 mmol/l), 4 nmol chloramphenicol (SIGMA), and for simulated transfection experiments 0.001 – 0.032 units of chloramphenicol acetyltransferase. After incubation (2 hours at 37 °C) an equal volume (145 µl) of acetonitrile (Merck) was added, vortexed thoroughly, and centrifuged for 10 min (9000 *g*). 20 µl of the supernatant were injected into the HPLC. A column, 4 mm diameter and 120 mm long packed with 5 µm C18 resin (Nucleosil, Macherey & Nagel) was used for HPLC. The column was equilibrated with aqueous methanol, volume fraction 0.5, which was also used as eluent. Pressure was 195 bar, flow rate 1.5 ml/min. UV absorption was measured at 275 nm (Gynko).

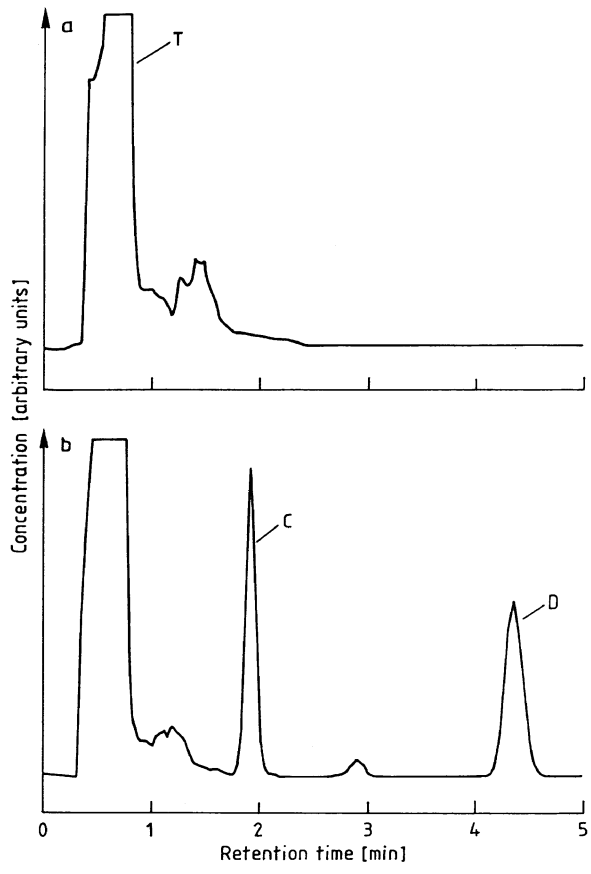

Fig. 1. Reference chromatogram
a. Cell homogenate in the absence of chloramphenicol and chloramphenicol diacetate. 'T' denotes peak due to Tris buffer.
b. 4 µmol of chloramphenicol and chloramphenicol diacetate added to homogenate (same as in a.). C' and 'D' denote peaks due to chloramphenicol and chloramphenicol diacetate, respectively.

Results and Discussion

Figure 1 shows reference chromatograms obtained with the homogenate in the absence of chloramphenicol (fig. 1a) and with defined amounts of chloramphenicol and chloramphenicol diacetate added (fig. 1b). UV-absorbing cellular material and buffer ingredients (Tris) elute between 0.3 min and 1.3 min. Chloramphenicol has a retention time of 1.9 min, its diacetylated form 4.4 min. A small peak eluting at 2.9 min is probably due to small amounts of monoacetylated chloramphenicol in the chloramphenicol diacetate stock. Even if a cell line should contribute more UV-absorbing material it will not interfere with the relevant peak of chloramphenicol monoacetate.

Figure 2 shows the results of simulated transfection experiments where increasing amounts of commercially available chloramphenicol acetyltransferase were added. Consequently, the peak of chloramphenicol decreases in correspondence to the increase of chloramphenicol monoacetete. Again the retention times of chloramphenicol peaks are not affected by any interference from cell homogenates.

The acetylation reaction of chloramphenicol is linear for 0.004 units of chloramphenicol acetyltransferase and 4 nmol of chloramphenicol. For higher amounts of chloramphenicol acetyltransferase, linearity can be obtained with higher concentrations of substrate (data not shown). The reactants are used in concentrations appropriate to the chloramphenicol acetyltransferase activity expected in transfected mammalian cells.

As expected from the results shown in figures 1 and 2 the small amount of chloramphenicol acetyltransferase activity present in transfected cells can indeed be analysed by direct loading of the HPLC (fig. 3).

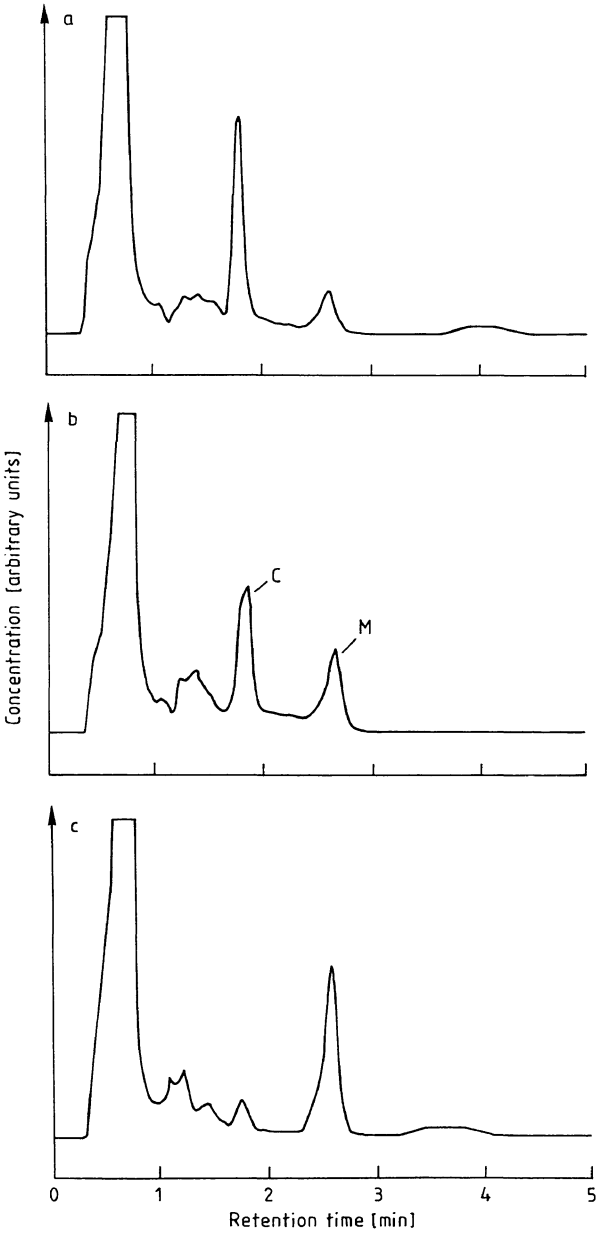

Fig. 2. Assay for chloramphenicol acetyltransferase activity in simulated transfection experiments.
Increasing amounts of chloramphenicol acetyltransferase were added to cell homogenates.
a. 0.002 units
b. 0.004 units
c. 0.016 units.
C' and 'M' denote peaks due to chloramphenicol and chloramphenicol monoacetate, respectively.

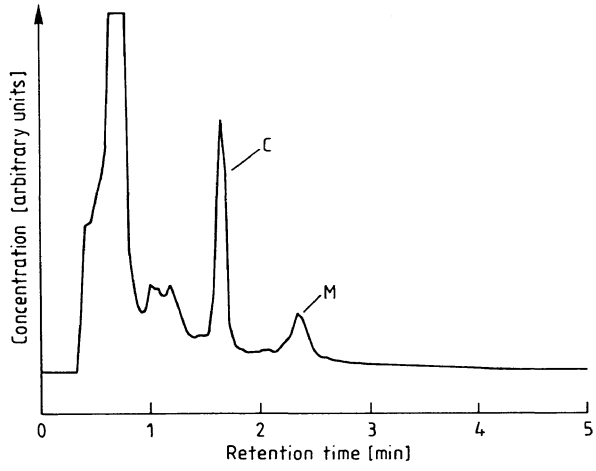

Fig. 3. Assay for chloramphenicol acetyltransferase activity in transfected mammalian cells.
'C' and 'M' denote peaks due to chloramphenicol and chloramphenicol monoacetate, respectively.

Cells were transfected with the plasmid pRSV CAT carrying the bacterial chloramphenicol acetyltransferase gene under the control of a promotor of *Rous* sarcoma virus. Only one fifth of the homogenate from $\sim 10^6$ cells was employed to produce the monoacetylated form of chloramphenicol. Thus, even the amounts of cells used with the conventional chloramphenicol acetyltransferase assay procedures yield sufficient peak sizes. In summary our procedure allows for quantitative as well as qualitative measurement of chloramphenicol acetyltransferase activity in transfected cells with a minimum of experimental steps involved. Results may be obtained within 3 hours. This is a fraction of the time needed for the radioactive chloramphenicol acetyltransferase assay.

Acknowledgement

This work was supported by Bundesministerium für Forschung und Technologie, by 'medac' (Project Transfektion...) and by "Trude Goerke Stiftung für Krebsforschung". We thank Dr. *G. Rossi* and Prof. *J.-H. Fuhrhop* for helpful advice, *W. Lamer* and *C. Zimmermann* of the Institut for Organische Chemie for help with HPLC methods and discussion of results.

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Horst Siegert
Institut für Molekularbiologie und Biochemie
Arnimallee 22
D-1000 Berlin 33